

THE EFFECT OF TESTICLE EXTRACT ON THE ROUS SARCOMA *

DONALD C. HOFFMAN, M.D., FREDERIC PARKER, JR., M.D., AND
THOMAS T. WALKER, M.D.

(From the Pathological Laboratory, Boston City Hospital, Boston, Mass.)

In recent publications, Duran-Reynals^{1, 2, 3} has shown that there exists in the testes of normal rabbits a substance, extractable in water or saline, which has the remarkable power of greatly enhancing skin lesions due to vaccine virus and to staphylococcus. This finding was confirmed by one of us⁴ working with vaccine virus and extending the work to determine the effect of this substance on the viruses of herpes, vesicular stomatitis and Borna disease. In each case the resultant infection was greatly enhanced. McClean,⁵ working with vaccine virus, reported similar results. Pijoan⁶ was able to enhance the lesions produced by twenty different bacteria by means of this substance. Ledingham and Barratt⁷ have referred to this enhancing agent as the "Reynals' factor." Hoffman and Duran-Reynals⁸ reported the fact that this extract failed to enhance the lesions produced by certain toxins and enzymes.

All the references thus far are concerned solely with the effect of testicle extract upon infections, toxins and enzymes. As to the effect of this substance on cell activity, there have been references to the stimulation of cell growth *in vitro* by testicle as well as other organ and tissue extracts.^{9, 10, 11} The effect of testicle extract on cell behavior *in vivo* is referred to in papers by Aievoli,¹² and Rho,¹³ who reported a stimulation of healing in chronic ulcers by means of such a substance.

On the other hand, Duran-Reynals¹⁴ recently reported that the Brown-Pearce rabbit tumor was greatly inhibited by incubating the tumor cell suspension with testicle extract at 37° C for from two to three hours prior to inoculation. This is the only reference that can be found dealing with the effect of testicle extract on tumor growth.

It was felt, therefore, that it would be of considerable interest to carry out an experiment to determine what effect, if any, this enhancing substance would have upon the Rous sarcoma (Chicken

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Tumor I). Since this tumor is practically the only one to grow from cell-free filtrates, and since there has been considerable question as to whether this is a true tumor in the sense of mammalian tumors or whether it is due to an infectious agent or enzyme, it was thought that such an experiment might shed some light on this much disputed subject.

EXPERIMENT I

Methods and Materials

The tumor used in these experiments was obtained through the courtesy of Dr. H. B. Andervont of the Harvard Medical School. The tumor supplied us was finely minced, taken up in a small amount of Locke's solution and injected intramuscularly into a young Plymouth Rock hen. After ten days the tumor had grown to a large size. The hen was killed and the tumor removed sterilely. It was then finely minced with scissors, passed through a food chopper and ground in a sterile mortar with sand. One portion of this tumor mass was diluted to a 1:5 suspension with Locke's solution and was called "tumor mash." The remainder was diluted 1:10 with Locke's solution, centrifuged, and the supernatant fluid passed through a Berkefeld N filter. This material was called "tumor filtrate."

Testicle extract was freshly prepared from testes of normal, healthy, adult rabbits. The testes were removed aseptically,* the fat was dissected off, and they were finely minced and ground with sterile sand. Sufficient Locke's solution was added to make a 1:10 suspension. This material was centrifuged at high speed for fifteen minutes and the supernatant fluid was passed through a Berkefeld N filter. This filtrate was tested for sterility before use.

Twelve Plymouth Rock chickens, 8 weeks old, were used in the experiment, each chicken receiving four intradermal inoculations in the skin of the breast. Before inoculation, mixtures consisting of equal parts of tumor mash and saline, tumor mash and testicle extract, tumor filtrate and saline, and tumor filtrate and testicle extract were prepared sterilely. The scheme of inoculation was as follows: upper left area received 0.5 cc. of the tumor mash-saline emulsion; upper right area received 0.5 cc. of the tumor mash-testis extract emulsion; lower left area received 0.5 cc. of the tumor

* All operations on animals were carried out under full ether anesthesia.

filtrate-saline suspension; and lower right area received 0.5 cc. of the tumor filtrate-testis extract suspension. Each inoculated area was marked off by painting a square about 2.5 by 2.5 cm. to 3 by 3 cm. around the site of injection with an alcoholic solution of gentian violet.

It was noted at the time of the inoculations that those mixtures containing testicle extract spread through the skin extremely rapidly, whereas those wheals containing only saline persisted for a considerable length of time.

Results

Four days after inoculation of the materials the chickens were examined for the first time. At that time, in each case, small tumor nodules had begun to form in the lower left and lower right regions, where tumor mash had been injected with testicle extract, and its control containing the same amount of mash plus saline. In each case except one, the tumor nodule resulting from the injection of mash plus testicle extract was from one-and-a-half to ten times as large as the corresponding control lesion. The areas which had received tumor filtrate had not shown tumor growth at that time. The tumors were measured every two days thereafter, and each time the areas which had received testicle extract in the inoculum showed much larger tumors than the controls. Those which had received tumor filtrate showed tumors for the first time ten days after inoculation. In each case with these tumors, the regions which had received testicle extract showed multiple small nodules which covered a considerably larger area than the corresponding controls. The fourteenth day after inoculation was the last day on which all chickens were still alive. The individual measurements and descriptions of the various tumors are recorded in tabular form in Table I.

It can be seen from the table that in each case the tumor resulting from the injection of mash plus testicle extract was larger than the corresponding control—in many cases markedly so. These tumors were rapidly growing and formed one nodule which was easy to measure in its two diameters. The approximate plane area of each nodule was determined and the average calculated for all the chickens. Based on average areas, the tumors resulting from mash plus testicle extract were 3.37 times as large as those arising from mash

plus saline. This is, of course, only an approximate figure and, if anything, shows less than the real enhancement phenomenon.

TABLE I
Description of Tumor Nodules 14 Days After Inoculation

Chicken No.	Tumor mash 0.25 cc. plus Saline 0.25 cc.	Tumor fil- trate 0.25 cc. plus Testicle ext. 0.25 cc.	Tumor filtrate 0.25 cc. plus Saline 0.25 cc.	Tumor filtrate 0.25 cc. plus Testicle ext. 0.25 cc.
	cm.	cm.		
1	1.5 x 1.7	2.3 x 2.0	3 small nodules	multiple nodules
2	3.0 x 1.5	6.0 x 4.0	few small nodules	multiple nodules filling marked area
3	3.5 x 2.0	5.0 x 4.0	few superficial and discrete nodules	same as No. 2 but ex- tending subcutaneously
4	1.5 x 1.3	2.5 x 2.0	few small discrete nodules	several small nodules
5	1.5 x 1.0	1.5 x 1.2	1 small subcutaneous nodule	1 small subcutaneous nodule
6	2.0 x 1.0	2.5 x 2.0	3 small nodules, larg- est 0.4 cm. diam.	several small nodules, largest 0.5 cm. diam.
7	3.5 x 2.0	5.0 x 4.0	few discrete nodules	multiple nodules filling marked area
8	2.0 x 1.7	5.0 x 4.0	few localized nodules	multiple small nodules
9	1.5 x 1.3	2.0 x 2.0	1 small nodule	multiple small nodules filling marked area
10	3.5 x 1.8	5.5 x 3.5	few localized nodules	multiple small nodules
11	2.0 x 1.5	3.5 x 4.0	1-2 small nodules	several nodules
12	1.5 x 1.2	2.5 x 2.0	2 small nodules	multiple nodules
Average area in square cm.	3.5	11.8

On the other hand, the tumors resulting from injection of tumor filtrate consisted in almost every case of several small nodules, either discrete or confluent. A fair measurement of these was not possible. Suffice it to say that those nodules occurring following injection of

tumor filtrate plus testicle extract, showed approximately the same degree of enhancement as the ones growing from tumor mash. The striking feature was the development of a multiplicity of small confluent nodules in the extract areas which was never observed in the case of the nodules arising from tumor filtrate plus saline.

After the death of the chickens, autopsies were performed on each and bits of the skin nodules were fixed in alcohol-formalin. Celloidin sections were made from each nodule and stained with hematoxylin and eosin. Each section showed typical Rous sarcoma, but no differences histologically could be made out in the various tumors. Most of the chickens showed metastatic nodules in lung and liver.

Thus it was seen that there had occurred marked enhancement in the growth of these tumors when rabbit testicle extract had been used as a diluent for the tumor mash or filtrate.

However, there seemed to be a possibility that this enhancement might have resulted either wholly or in part from the presence of foreign protein (rabbit protein) rather than the testicle extract principle itself. Also, the question arose as to whether extract of rooster testis would exercise the same effect as rabbit testis. Consequently, a second experiment was designed to clarify these points.

EXPERIMENT II

Methods and Materials

The chicken tumor used in the second experiment was likewise procured fresh from Dr. Andervont at the Harvard Medical School and inoculated into the breast muscle of a young Plymouth Rock hen. After ten days, the hen was killed and the tumor removed aseptically. The procedure for preparing the tumor injection material was the same as in the first experiment with the exception that no tumor mash was used and the filtrate dilution was 1:5.

The same rabbit testicle extract was used as in Experiment I.

Rooster testicle extract was made in exactly the same manner as rabbit testicle extract.

Normal rabbit serum was obtained by bleeding a normal rabbit from the heart and collecting the blood in a sterile tube. After it had clotted and the clot separated, the tube was centrifuged at high speed for twenty minutes and the serum was pipetted off.

As in the first experiment the injection masses were prepared before inoculation as follows: mixtures of equal parts of tumor filtrate and Locke's solution were made in one tube, equal parts of tumor filtrate and rooster testicle extract in another, equal parts of tumor filtrate and rabbit serum in another, and equal parts of tumor filtrate and rabbit testicle extract in the last.

Ten healthy young Plymouth Rock roosters, 8 weeks old, were used. Each received four intradermal injections according to the following scheme: upper left area, 0.5 cc. of the filtrate-Locke's solution mixture; lower left area, 0.5 cc. of the filtrate-rooster testicle extract mixture; upper right area, 0.5 cc. of the filtrate-rabbit serum mixture; and lower right area, 0.5 cc. of the filtrate-rabbit testicle extract mixture.

At the time of the inoculations it was observed that the mixture which contained rabbit testicle extract spread very rapidly through the tissues, whereas the other three mixtures left a wheal for an appreciable length of time.

Results

The first observations were made eight days after inoculation. By this time every chicken had developed tumors in each area. In each instance but one, the tumors growing after inoculation of filtrate plus rabbit testicle extract were considerably larger than the Locke's solution control. In the other areas no such constant difference in the size of the tumor nodules was apparent. In practically every case the control area was the smallest.

The tumors were measured at two-day intervals and the thirteenth day after injection was the last day when all animals were still alive. The tumors were growing rapidly, and this day was selected for recording the measurements in tabular form. These appear in Table II.

As in the first experiment, the tumors resulting from the injection of tumor filtrate plus rabbit testicle extract were in each case larger than the Locke's solution control tumors. In addition they were also larger than the rooster testicle extract and rabbit serum tumors. In want of a better method of getting an average size for comparison, the plane areas were again computed and averaged. As can be seen from the table the average area of the tumors resulting from injection of tumor filtrate plus rabbit testicle extract was 12.67 square

cm., whereas the Locke's solution control tumors averaged 3.9 square cm. The rooster testicle extract tumors averaged 3.2 square

TABLE II
Description of Tumor Nodules 13 Days After Inoculation

Chicken No.	Tumor filtrate 0.25 cc. plus Locke's sol. 0.25 cc.	Tumor filtrate 0.25 cc. plus Rooster testicle ext. 0.25 cc.	Tumor filtrate 0.25 cc. plus Rabbit serum 0.25 cc.	Tumor filtrate 0.25 cc. plus Rabbit testicle ext. 0.25 cc.
13	cm. 2.5 x 2.0	cm. 3.0 x 2.0	cm. 2.6 x 3.0	cm. 5.0 x 3.5
14	1.5 x 1.3	0.9 x 1.0	1.7 x 2.1	deep extension 5.0 x 5.0
15	3.7 x 2.0	3.0 x 1.4	2.4 x 1.9	deep extension 6.4 x 2.3
16	2.8 x 2.0	2.8 x 1.8	2.7 x 2.5	4.0 x 2.7
17	0.4 x 0.4	1.4 x 0.4	2.2 x 2.2	deep and diffuse 5.5 x 2.8
18	3.2 x 1.5	2.3 x 1.4	3.0 x 3.0	deep extension 3.5 x 4.3
19	0.5 x 0.5	0	1.0 x 1.0 0.2 x 0.2	3.0 x 1.7
20	1.5 x 1.3	2.5 x 1.2	7 confluent nod- ules, largest 0.7 x 0.6	large confluent nod- ules, deep exten- sion 2.5 x 1.8
21	2.5 x 2.1	2.8 x 2.0	2.8 x 2.8	ulcerated 4.0 x 3.2
22	2.0 x 1.9	2.5 x 1.4	deep extension 2.3 x 1.7	deep extension 4.0 x 2.5
Average area in square cm.	3.9	3.2	5.05	12.67

cm., and the rabbit serum tumors averaged 5.05 square cm. It would seem, therefore, that the mere presence of rabbit protein produced a considerable enhancement of the tumors, but not nearly so strikingly and constantly as did the rabbit testicle extract.

DISCUSSION

The experiments here reported show clearly that rabbit testicle extract enhances quite markedly the growth of the Rous sarcoma (Chicken Tumor I), following its injection together with the tumor-producing agent. This enhancement occurs equally well following inoculation of cell-free filtrate of the tumor or with injection of a mash consisting of tumor cells in suspension. In the latter case the resultant tumors appeared much more quickly after inoculation, but the degree of enhancement was essentially the same as that following injection of the filtrate. The tumors arising from tumor mash plus rabbit testicle extract, using the average area of the tumors on the fourteenth day, were 3.37 times the average size of the tumors resulting from injection of tumor mash plus saline. The tumors which resulted from injection of the Berkefeld filtrate plus rabbit testicle extract based on average areas on the thirteenth day were 3.25 times the size of those arising from filtrate plus Locke's solution. Thus it will be seen that the degree of enhancement using rabbit testicle extract was practically the same in two different experiments using tumor mash in one instance and cell-free filtrate in the other.

An interesting finding was the fact that rooster testicle extract prepared under exactly the same condition as rabbit testicle extract was wholly without this enhancing property. Just why this should be true is not clear and we shall not attempt to offer an explanation. However, it was noted at the time of inoculation that the wheals caused by intradermal injections disappeared surprisingly rapidly when rabbit testicle extract was a part of the injection mass, whereas the wheals persisted for a considerable length of time when rooster testicle extract was used.

That normal rabbit serum is not responsible for this enhancement is clearly shown in the second experiment. There was a slight degree of enhancement, as indicated by the average tumor sizes on the thirteenth day. Those tumors which arose from tumor filtrate plus rabbit serum were 1.3 times the size of the Locke's solution control tumors. This is in marked contrast to the enhancement of the tumors by rabbit testicle extract, which not only increased in two plane dimensions, but often protruded and practically always invaded the deep tissues.

It is beyond the scope of this brief communication to attempt to theorize about the mechanism of this remarkable phenomenon. We merely present the experimental facts. For a discussion of this subject, the reader is referred to recent publications by McClean⁵ and by Hoffman and Duran-Reynals.⁸

The question naturally arises as to whether this tumor behaves like a true tumor in the sense of mammalian tumors, or whether it is the manifestation of a virus disease, an infectious granulomatous process, or an enzyme-like substance. Without attempting to make any statement which would align us on one side or the other of this controversy, we merely point out the fact that the tumor is greatly enhanced by this agent, which, so far as we can say at present, enhances only virus or bacterial infections. That this tumor is not due to a bacterial agent is pretty well accepted at this time. Enzymes are apparently not enhanced by this substance, and the only experimental work on mammalian tumors thus far reported is that by Duran-Reynals¹⁴ who showed that a rabbit tumor of epithelial origin is actually inhibited by this substance. It is only fair, however, to point out that in the experimental work of Duran-Reynals on the rabbit tumor, the tumor mash plus testicle extract was incubated for from two to three hours at 37° C before injection. This technique was not followed by us. However, our injection masses were made up approximately one to two hours before inoculation and stood at room temperature during that time.

Thus there may or may not be evidence hinting that this tumor falls in the class of diseases due to infectious agents. Before we are definitely willing to say it is so, we should like to see considerable more work done on the effect of this enhancing agent.

CONCLUSIONS

1. Rabbit testicle extract markedly enhances growth of the Rous sarcoma (Chicken Tumor I) in chickens.
2. This effect is the same whether tumor mash or a cell-free filtrate of the tumor is used in the inoculations.
3. Rooster testicle extract causes no enhancement.
4. Normal rabbit serum causes a slight degree of enhancement.

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